

Ectomycorrhizal Inoculation of Containerized Douglas-fir and Lodgepole Pine Seedlings With Six Isolates of *Pisolithus tinctorius*

RANDY MOLINA

ABSTRACT. Containerized Douglas-fir and lodgepole pine seedlings grown under lowered fertility were inoculated with vegetative mycelium from six isolates of the ectomycorrhizal fungus *Pisolithus tinctorius*. The isolates differed both culturally and in effectiveness as ectomycorrhizal inoculum. The percent of seedlings successfully inoculated and percent of feeder roots colonized differed significantly among the isolates. Analysis of variance, however, for seedling height, stem diameter, stem and root dry weights, and top/root ratio showed no significant differences between any inoculation treatment and controls for either tree species. This study reinforces the concept that a wide array of fungus ecotypes should be tested before a specific strain is selected for wide-scale nursery inoculations. FOREST SCI. 25:585-590.

ADDITIONAL KEY WORDS. *Pseudotsuga menziesii*, *Pinus contorta*, forest regeneration.

AN ESTIMATED 76 MILLION containerized seedlings were produced in Oregon and Washington in 1977 (Stein and Owston 1977). Cultural practices used in producing containerized seedlings, e.g., artificial (nonsoil) substrates, frequent doses of concentrated soluble fertilizers, and greenhouse rearing, however, often restrict development of normal mycorrhizal root systems. Most of the seedlings we have examined from various Northwest container nurseries routinely lack mycorrhizae. If containerized seedlings can be produced with well-developed mycorrhizal root systems, their field survival and growth may be greatly improved, especially on sites hard to regenerate (Marx and Barnett 1975).

Bare root nursery stock can now be effectively inoculated with selected isolates of beneficial ectomycorrhizal fungi (Marx and Bryan 1975); similar techniques have succeeded with container grown stock (Marx and Barnett 1975, Ruehle and Marx 1977). The ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker and Couch appears well suited for artificial inoculation. *P. tinctorius* inoculated seedlings have been shown to significantly outperform uninoculated seedlings both in the nursery (Marx and Bryan 1975, Marx and others 1976) and in plantations on severely disturbed sites (Marx 1976) and on more routine reforestation sites (Marx and others 1977). Its worldwide distribution and broad host range further augment its potential to benefit forestry programs around the world (Marx 1977).

Trappe (1977) discusses the strong ecotypic variation expressed between isolates of a single ectomycorrhizal fungus both culturally and in their effects on host metabolism. He stresses the need to test many ecotypes of a particular fungus in selecting specific isolates for widespread nursery inoculations. The purposes of this study were to examine the feasibility of artificially inoculating containerized Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and lodgepole pine (*Pinus contorta* Dougl.) seedlings with six isolates of *P. tinctorius* under routine cultural conditions, and to compare effects of these isolates upon seedling growth.

The author is a botanist at USDA Forest Service, Pacific Northwest Forest and Range Experiment Station, Portland, Oregon 97208. Manuscript received 18 January 1979.

TABLE 1. Radial colony growth and color, and sources of *Pisolithus tinctorius* isolates used for inoculation of container Douglas-fir and lodgepole pine seedlings.

Isolate no.	Forest type and location	Colony color	Radial growth ^a mm
S-210	<i>Pinus taeda</i> Georgia	light brown	86.1 a
S-216	<i>Pseudotsuga menziesii</i> Coos Co., Oregon	bright yellow	50.4 b
S-360	<i>Lithocarpus densiflorus</i> <i>Pinus ponderosa</i> <i>Arbutus menziesii</i> Siskiyou Co., California	bright yellow	43.4 bc
A-172	<i>Quercus garryana</i> Corvallis, Oregon	light brown	37.6 bc
S-310	<i>Lithocarpus densiflorus</i> <i>Pseudotsuga menziesii</i> <i>Pinus ponderosa</i> <i>Arbutus menziesii</i> Siskiyou Co., California	bright yellow	29.9 c
S-371	<i>Pseudotsuga menziesii</i> <i>Pinus ponderosa</i> Siskiyou Co., California	bright yellow	26.4 c

^a Isolates not sharing a common letter differ significantly ($P < 0.05$) by Scheffé test for differences among treatment means.

MATERIALS AND METHODS

Pisolithus tinctorius Isolates.—Table 1 lists the six *P. tinctorius* isolates used in this experiment. All had been originally isolated from sporocarp tissue. Isolates S-216, S-310, S-360, and S-371 were native to southwest Oregon-Northern California forest habitats and were isolated in late fall 1976. Isolate A-172 sporocarp was found fruiting beneath an ornamental *Quercus* species in Corvallis, Oregon (nonforest habitat) and was also isolated in fall 1976. Isolate S-210 was obtained from Dr. D. H. Marx, Athens, Georgia, in summer 1976 and originally was isolated in association with loblolly pine in 1967. It has subsequently been reisolated from mycorrhizae every 1 or 2 years prior to our receipt (D. H. Marx, personal communication). All isolates were stored on modified Melin-Norkrans agar substrate (MMN) (Marx 1969) with glucose substituted for sucrose and transferred every 3 mo prior to inoculum preparation.

To assess growth differences among the isolates, each was initially grown for 21 days on MMN agar in 9 cm diam glass petri plates. For each isolate, ten 5 mm diam inoculum disks were then cut and removed from the colony edge and placed one each into ten replicate plates also containing MMN agar. All plates were then grown in the dark at 20°C. After 30 days, colony diameters were measured to the nearest mm and the results subjected to analysis of variance.

Inoculum Preparation.—All isolates were initially grown for 4 weeks at 20°C in 200-ml capped bottles partially filled with MMN nutrient solution and small chips of broken glass. Each bottle was shaken twice weekly to fragment the actively growing mycelium. Vegetative mycelial inoculum was prepared similar to pro-

cedures described by Marx and Bryan (1975). Glass capped 2-liter flasks filled with 1,450 ml of vermiculite plus 50 ml of sphagnum peat moss were moistened with 800 ml of MMN solution and autoclaved for 1 hr at 120°C. Flasks were then aseptically inoculated with 50 ml of mycelial slurry. Control flasks were left uninoculated. Two flasks were prepared for each isolate. After 11 weeks growth at room temperature, inoculum was removed from the flasks and leached with approximately 15 times its volume of cold running tap water to remove unused nutrients. To minimize disturbance of the vermiculite particles, excess water was removed from particles by suction filtration. Inoculum was placed into plastic bags and stored overnight at 5°C.

INOCULATION AND SOWING

A 1:1 peat moss-vermiculite mixture, steam pasteurized at 80°C for 30 min, was used as the potting substrate. To evenly distribute the vegetative inoculum, one part of inoculum was vigorously mixed with six parts of substrate in large plastic bags. Single celled "Leach tube" containers, 65-ml capacity, were then filled with the inoculated potting substrate and triple sown with stratified Douglas-fir or lodgepole pine seeds. Sixty sown cells of each tree-fungus combination were randomly distributed into each of three replicate blocks of 20 cells each. All treatments were separated by one blank cell row to minimize contamination between isolates. Seeds were misted for 5 min twice daily until germination was complete. All cells were then thinned to one seedling each.

Growing Conditions.—Seedlings were greenhouse-reared from early June through November 1977. Supplemental light of approximately 11,000 lx over a 15-h photoperiod was provided by two overhead sodium-vapor lamps. To approximate current nursery practices, fertilization rates and schedules generally followed those suggested by Owston (1975) for container-grown western conifers. Because high fertilizer rates commonly used in container nurseries are known to retard mycorrhizal colonization by *P. tinctorius* (Marx and Barnett 1975), approximately one-half the recommended dosage was applied. A completely soluble 20-19-18 NPK fertilizer (Peat-lite special) was dissolved in tap water and evenly distributed manually over all seedlings at the rate of 12 g/m² of bench space. Each seedling thus received approximately 6.2 mg of fertilizer. Sequestrene 300 iron chelate was added simultaneously at the rate of 3 g/m² of bench space or approximately 1.6 mg/seedling. Fertilizer was added weekly from July through October. Seedlings were watered with tap water as needed. Watering was reduced in the fall to induce bud set.

Data Collection.—At the end of the experiment, all seedlings were harvested and their roots gently washed free of potting substrate. Each root system was microscopically examined for mycorrhizal colonization by *P. tinctorius*. From among seedlings with only *P. tinctorius* ectomycorrhizae, five were randomly selected from each treatment replication, and seedling heights, stem diameters, and oven-dry weights of tops and roots were recorded. The degree of mycorrhiza formation was visually estimated to the nearest 10 percent of total feeder roots. Control seedlings or *P. tinctorius*-inoculated seedlings with other, contaminant mycorrhiza types present were not included in any analysis. All results were then subjected to analysis of variance, and differences between treatment means were compared with Scheffé tests.

RESULTS

Cultural Characteristics.—Marked differences in color and radial growth occurred among the six isolates tested (Table 1). All isolates from Northwest forest

TABLE 2. Percent of container Douglas-fir and lodgepole pine seedlings successfully inoculated with six isolates of *Pisolithus tinctorius*.

Isolate no.	Mean percent of seedlings successfully inoculated		
	Douglas-fir	Lodgepole pine	\bar{x}^a —Douglas-fir + lodgepole pine
A-172	92.3	86.6	89.5 a
S-210	84.1	94.7	89.4 ab
S-216	47.2	74.6	60.9 abc
S-371	17.9	40.0	28.9 c
S-360	15.5	33.3	24.4 c
S-310	2.0	8.6	5.3 d

^a Because there was no significant difference in the analysis of variance between the two tree species, means averaged over the two tree species were compared. Those means not sharing a common letter differ significantly ($P < 0.05$) by Scheffé test.

habitats were bright yellow; the Georgia isolate (S-210) and the Corvallis isolate (A-172) were light brown. These colors remained constant in agar and peat moss-vermiculite inoculum substrates.

The Georgia isolate had significantly greater radial growth ($P < 0.01$) than all the Northwest isolates; with the exception of isolate S-216, it grew nearly twice as fast as all other isolates (Table 1). Significant differences in radial growth ($P < 0.05$) remained, however, between the Northwest isolates, indicating ecotypic variation.

All isolates had completely permeated the inoculum substrate after 11 weeks. Although not quantified, differences in mycelium density were visually apparent. Isolates S-210 and S-216 were most dense and displayed abundant aerial mycelium above the inoculum substrate. S-360, S-310, and S-371 grew less dense and without appreciable aerial mycelium. A-172 grew diffusely throughout the substrate.

Mycorrhizal Formation.—All isolates tested formed ectomycorrhizae with both Douglas-fir and lodgepole pine. Mantle colors were similar to pure mycelial cultures in all cases; isolates S-210 and A-172 had pale golden brown ectomycorrhizae while all the Northwest forest isolates were bright yellow. A mixture of golden brown and bright yellow ectomycorrhizae was not seen on any seedlings, indicating a lack of cross contamination between treatments.

The percent of seedlings successfully inoculated differed significantly ($P < 0.05$) among the six isolates (Table 2). The two pale brown isolates were consistent in this regard and short roots ectomycorrhizal ranged from 84 to 95 percent. Isolates S-216, S-360, and S-371 were less consistent with intermediate to low percent inoculation success. Isolate S-310 formed mycorrhizae with very few seedlings and was excluded from further statistical analysis. Inoculation success was generally but not significantly greater for lodgepole pine than for Douglas-fir due to large variation between replicates.

Significant differences ($P < 0.05$) also occurred among isolates in the percent of feeder roots colonized (Table 3). When data on percent feeder roots ectomycorrhizal for Douglas-fir were averaged together with lodgepole pine, isolates S-210 and S-216 colonized approximately 75 percent of the roots. On many individual seedlings, as many as 95 percent of the total feeder roots were colonized by these two isolates. Isolates S-360 and S-371 colonized about 45 percent of the feeder roots. Occasional seedlings from these treatments, however, were also observed to have upwards to 95 percent of feeder roots colonized. Although

TABLE 3. Percent mycorrhiza formation of container Douglas-fir and lodgepole pine seedlings inoculated with six isolates of *Pisolithus tinctorius*.

Isolate no.	Mean percent of total feeder roots colonized		
	Douglas-fir	Lodgepole pine	\bar{x}^a —Douglas-fir + lodgepole pine
S-216	75.6	76.0	75.8 a
S-210	65.3	84.0	74.7 a
S-360	56.9	34.7	45.8 b
S-371	46.7	43.1	44.9 b
A-172	19.1	33.3	26.2 b

^a Because there was no significant difference in the analysis of variance between the two tree species, means averaged over the two tree species were compared. Those means not sharing a common letter differ significantly ($P < 0.05$) by Scheffé test.

isolate A-172 successfully colonized the greatest number of seedlings, the percent of feeder roots colonized was generally low, averaging 19 percent for Douglas-fir and 33 percent for lodgepole pine. Some isolates appeared to perform better on one conifer species than on the other, but the differences were not significant. Control seedlings were relatively free of any mycorrhizal colonization. Approximately 5 percent of control and *P. tinctorius* seedlings had moderate infections of *Thelephora americana*. These seedlings were not included in any statistical tests.

Seedling Growth.—No visual differences in seedling growth were obvious between treatments in the greenhouse. Analysis of variance for seedling height, stem diameter, top and root dry weights, and top/root ratio showed no significant differences between any inoculation treatment and controls for either tree species. Douglas-fir seedlings ranged from 12.7 to 14.2 cm in height, 1.9 to 2.0 mm in stem diameter, 0.46 to 0.55 g in stem dry weight, and 0.40 to 0.47 g in root dry weight over the six treatments. Lodgepole pine seedlings ranged from 8.4 to 10.0 cm in height, 1.6 to 1.8 mm in stem diameter, 0.38 to 0.50 g in stem dry weight, and 0.28 to 0.41 g in root dry weight over the six treatments. Seedlings appeared healthy and without nutrient deficiencies. Seedlings were slightly smaller, however, particularly in stem diameter, than the suggested target size for plantable container stock in Northwest forests (Cleary and others 1978, Owston 1975).

DISCUSSION

As determined by Marx and Barnett (1975) and Ruehle and Marx (1977) for container-grown loblolly pine seedlings, ectomycorrhizal inoculation of Douglas-fir and lodgepole pine containerized seedlings with pure cultures of *P. tinctorius* is feasible. Very little change in routine nursery practice is needed except in fertility management. In container operations where roots are frequently bathed with high doses of completely soluble fertilizers of twice to four times the concentration used in this study (Owston 1975), ectomycorrhiza formation is greatly impaired. In this study, with approximately half the recommended fertilizer regime applied, ectomycorrhizal inoculation was successful. Yet, uninoculated control seedlings grew equally well. Obviously, even at this reduced fertility level, readily available nutrients were adequate for comparable growth of nonmycorrhizal control seedlings. The lowered fertility, however, also produced seedlings slightly below the target sizes for plantable stock. Target size may have to be

adjusted after field trials with mycorrhizal seedlings. It is quite possible that smaller mycorrhizal seedlings will outperform larger nonmycorrhizal ones.

The fungus isolates differed both culturally and in effectiveness as ectomycorrhizal inoculum. This study reinforces the concept that a wide array of ecotypes should be tested before a specific strain is selected for widespread nursery inoculations (Trappe 1977). The age of the isolates, their original host associations, and their reisolation from mycorrhizal syntheses may significantly influence their ability to synthesize mycorrhizae on certain hosts. Trappe (1977) states that D. H. Marx (personal communication) found 20 isolates of *P. tinctorius* exhibited considerable variation in ability to synthesize mycorrhizae on southern pines. The Georgia isolate of *P. tinctorius*, which has undergone intense screening and testing for overall effectiveness, performed as well if not better than all the Northwest isolates tested. Isolate S-216 from southwest Oregon, however, compared favorably in colonizing root systems although it had lower overall inoculation success. The final and most important test to evaluate effectiveness of any mycorrhizal inoculum is how inoculated seedlings perform compared to uninoculated stock after outplanting. Nurserymen will not want to inoculate seedlings unless it significantly improves nursery production or field performance. Continuing efforts to select beneficial fungi adapted to nursery practices as well as outplanting sites are currently in progress.

LITERATURE CITED

CLEARY, B. D., R. D. GREAVES, and P. W. OWSTON. 1978. Chapter 6, Seedlings. In Regenerating Oregon's Forests (B. D. Cleary, R. D. Greaves, and R. K. Hermann, eds), p 63-97. Sch For, Oreg State Univ, Corvallis.

MARX, D. H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* 59:153-163.

MARX, D. H. 1976. Use of specific mycorrhizal fungi on tree roots for forestation on disturbed surface areas. In Proc Conf on Forestation of Disturbed Areas, Birmingham, Ala., April 14-15, 1976 (K. A. Utz, ed), p 47-65. USDA Forest Serv, Atlanta. 76 p.

MARX, D. H. 1977. Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. *Can J Microbiol* 23:217-223.

MARX, D. H., and J. P. BARNETT. 1975. Mycorrhizae and containerized forest tree seedlings. In Proc North Am Containerized Forest Tree Seedling Symp (R. W. Tinus, W. I. Stein, and W. E. Balmer, eds), p 85-92. Great Plains Agric Counc Publ 68, 458 p.

MARX, D. H., and W. C. BRYAN. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. *Forest Sci* 21:245-254.

MARX, D. H., W. C. BRYAN, and C. E. CORDELL. 1976. Growth and ectomycorrhizal development of pine seedlings in nursery soils infested with the fungal symbiont *Pisolithus tinctorius*. *Forest Sci* 22:91-100.

MARX, D. H., W. C. BRYAN, and C. E. CORDELL. 1977. Survival and growth of pine seedlings with *Pisolithus* ectomycorrhizae after two years on reforestation sites in North Carolina and Florida. *Forest Sci* 23:363-373.

OWSTON, P. W. 1975. Two-crop production of western conifers. In Proc North Am Containerized Forest Tree Seedling Symp (R. W. Tinus, W. I. Stein, and W. E. Balmer, eds), p 104-111. Great Plains Agric Counc Publ 68, 458 p.

RUEHLE, J. L., and D. H. MARX. 1977. Developing ectomycorrhizae on containerized pine seedlings. USDA Forest Serv Res Note SE-242, 8 p. Southeast Forest Exp Stn, Asheville, N.C.

STEIN, W. I., and P. W. OWSTON. 1977. Containerized seedlings in western reforestation. *J For* 75:575-578.

TRAPPE, J. M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. *Annu Rev Phytopathol* 15:203-222.